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<p>(54) Title: TNF-BETA-LIKE PROTEIN FOR TREATING PROSTATE CANCER, AND RELATED NUCLEIC ACID MOLECULES, PHARMACEUTICAL COMPOSITIONS AND METHODS</p> <p style="text-align: center;"><u>NUCLEOTIDE SEQUENCE OF GF-2H</u></p> <pre> 1 CGAGTCCCAAG CTCTGAGCCG CAACCTGCAC AGCGATGCC GGGCAAGAAC 51 TCAGGACGCT GAATGGCTC CAGATGCTC TGGTGTGCT GGTGCTCTCG 101 TGGCTCCCGC ATGGGGGGCGC CCTGTCCTCG GCGCAGGGCA GCGCGCGCAAG 151 TTTCCCGGGA CCCTCAGAGT TGCACCTCCGA AGACTCCAGA TTCCGAGAGT 201 TGCAGGAACCG CTACGAGGAC CTGCTAACCA GGCTGCGGGC CAACCAGAGC 251 TGGGAAGATT CGAACACCGA CCTCGTCCCCG GCCCCTTCAG TCCGGATACT 301 CACGCCAGAA GTGCGGCTGG GATCCGGCGG CCACCTGCAC CTGCGTATCT 351 CTCGGGCCGC CCTTCCTGCG GGGCTCCCCG AGGCCTCCCG CCTTCACCGG 401 GCTCTGTTCC GGCTGTCCCC GACGGCGTCA AGGTCGTTGGG ACGTGACACG 451 ACCGCTGCGG CGTAGCTCA GCCTTGCAAG ACCCCCAGGCG CCCGCGCTGC 501 ACCTGCGACT GTCGCCGCCG CGCTCGCAGT CGGACCAAAT GCTGGAGAAA 551 TCTTCGTCGG CACGGCCCCA GCTGGAGTTG CACTTGCAGG CGCAAGCGC 601 CAGGGGGCGC CGCAGAGCGC GTGCGCGAA CGGGGACCCAC TGTCCGCTCG 651 GGCCCGGGCG TTGCTGCCGT CTGCACACGG TCCCGCGTC GCTGGAAAGAC 701 CTGGGCTGGG CGCATTTGGGT GCTGTCGCCA CGGGAGGTGCA AAGTGACCAT 751 GTGCATCGGC GCGTGGCCGA GCCAGTTCCG GGGGGCAAAAC ATGCAACGCG 801 AGATCAAGAC GAGCCTGCA CGCCTGAAGC CGCACACGGT GCCAGCGCCC 851 TGCTCGTGC CGCCCAAGCTA CAATCCCATG GTGCTCATTC AAAAGACCGA 901 CACCGGGGTG TCGCTCCAGA CCTATGATGA CTTGTTAGCC AAAGACTGCC 951 ACTGCATATG AGCAGTCCCTG GTCCCTCCAC TGTGCAACCTG CGCGGGGGAG 1001 GCGACCTCAG TTGTCCTGCC CTGTGGAATG GGCTCAAGGT TCCTGAGACA 1051 CCCGATTCCCT GCCCAAACAG CTGTATTTAT TAAAAACTCT GATGATAAAA 1101 ATAAAGCTTG TCTTGAATG TT </pre> <p>(57) Abstract</p> <p>This invention provides proteins which selectively inhibit the proliferation of androgen-independent prostate cancer cells. This invention also provides a nucleic acid molecule encoding the instant proteins, including a nucleic acid molecule suitable for use in gene therapy for treating a subject afflicted with androgen-independent prostate cancer. This invention further provides a composition of matter which selectively kills androgen-independent prostate cancer cells, and comprises the instant proteins and a toxic moiety operably affixed thereto. Finally, the instant invention provides related pharmaceutical compositions and methods for treating androgen-independent prostate cancer.</p>			

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TNF-BETA-LIKE PROTEIN FOR TREATING PROSTATE CANCER, AND RELATED NUCLEIC ACID MOLECULES, PHARMACEUTICAL COMPOSITIONS AND METHODS

Field of the Invention

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This invention relates to proteins useful for treating subjects suffering from androgen-independent prostate cancer. This invention also relates to nucleic acid molecules, pharmaceutical compositions and methods for treating subjects 10 with this disorder.

Background of the Invention

Throughout this application, various publications are 15 cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

Carcinoma of the prostate ("prostate cancer") is one of the 20 most common types of malignancy. Among men 50 years of age or older, 30% harbor foci of cancer within the prostate. Only 1% of these afflicted men will be properly diagnosed with prostate cancer each year, highlighting a discrepancy between the high incidence of disease as revealed at autopsy and the much lower 25 incidence revealed clinically. In 1992, 134,000 men in the United States were diagnosed with prostate cancer, and 32,000 died from this disease. Of all men clinically diagnosed with prostate cancer, more than half die within 10 years, and more than two-thirds suffer local or systemic progression despite 30 therapy. Recently, testing for serum prostate-specific antigen (PSA) has allowed earlier detection, but it is unclear what the natural progression of the disease is in patients with elevated PSA but without large tumor burdens.

35 For cancer confined to the prostate, primary radiation or radical prostatectomy have roughly equivalent efficacy. Recurrences can be treated with radiation for primary surgery and, with difficulty, surgery for primary radiotherapy.

Metastatic prostate lesions predominantly go to bone and can be treated by decreasing the androgen level in the subject. This can be done chemically by blocking adrenal and gonadal androgen production with drugs, or surgically by castration (which does not actually block adrenal production). The observation that patients will respond to such hormonal manipulation for only 6 months or so and then worsen suggests that the metastatic prostate lesions can become androgen-independent. This suggestion is supported by the finding that cultured cell lines from prostatic carcinomas contain mixed clones. These mixed clones are of three types: androgen-dependent, which survive only in the presence of exogenous androgen; androgen-sensitive, which remain alive in the absence of androgen and proliferate in its presence; and androgen-independent, which can survive as well as proliferate in the absence of androgens.

Among subjects suffering from prostate cancer, a significant population display clinical androgen-independence, i.e., the presence of prostate cancer cells which continue to proliferate even after therapeutic elimination of androgens. Surgery to remove a prostate tumor mass often results in the removal of androgen-independent cells along with other types of cells. However, surgery alone is rarely effective in this regard, since at least some androgen-independent cells remain. Accordingly, there exists a strong -- and unmet -- need for methods of treating prostate cancer using pharmaceutical agents which specifically inhibit the proliferation of androgen-independent cancer cells.

Summary of the Invention

This invention provides a recombinant protein and an isolated protein, each of which selectively inhibits the proliferation of androgen-independent prostate cancer cells when contacted therewith under suitable conditions, and whose amino acid sequence comprises the amino acid sequence shown in Figure 2, or functional derivative thereof.

10 This invention further provides a pharmaceutical composition for treating a subject afflicted with androgen-independent prostate cancer, comprising one of the instant proteins and a pharmaceutically acceptable carrier.

15 This invention further provides a nucleic acid molecule consisting essentially of a nucleic acid sequence encoding the amino acid sequence of Figure 4 or functional derivative thereof, including, but not limited to, a nucleic acid molecule suitable for use in gene therapy for treating a subject
20 afflicted with androgen-independent prostate cancer.

This invention further provides a pharmaceutical composition for treating a subject afflicted with androgen-independent prostate cancer, comprising the instant nucleic acid
25 molecule suitable for use as a gene therapy vector and a pharmaceutically acceptable carrier.

This invention provides a composition of matter which selectively kills androgen-independent prostate cancer cells
30 when contacted therewith under suitable conditions, and which comprises the instant protein and a toxic moiety operably affixed thereto.

This invention also provides a pharmaceutical composition
35 for treating a subject afflicted with androgen-independent prostate cancer, comprising the instant composition of matter and a pharmaceutically acceptable carrier.

Finally, this invention provides methods of treating a subject afflicted with androgen-independent prostate cancer which comprise administering to the subject a therapeutically effective dose of one of the instant pharmaceutical compositions.

Brief Description of the Figures

Figure 1 shows the DNA sequence of GF-2H.

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Figure 2A shows a comparison between the amino acid sequence of mature GF-2H protein (identified as "ACTIVE2H2") and those of other members of the TGF-beta family.

10 Figure 2B shows the amino acid sequence of the immature GF-2H protein.

Figure 3A shows a purification scheme of GF-2H.

15 Figure 3B shows a purification scheme of rGF-2H.

Figure 4 shows the amino acid sequence of mature GF-2H.

20 Figure 5 shows the expression of GF-2H-encoding mRNA in different tissues.

Figure 6 shows the modulation of prostate cancer cell line PC-3 by mature GF-2H.

25 Figure 7 shows the modulation of prostate cancer cell line PC-3 by mature TGF-beta.

Detailed Description of the Invention

Transforming growth factor type-beta (TGF-beta) represents a large family of multifunctional factors having diverse activities. TGF-beta is prototypic of a superfamily of growth, differentiation, and morphogenesis factors, such as inhibins, activins, Mullerian inhibiting substance, decapentaplegic product, and TGF-beta2. These factors are all structurally related to TGF-beta. Additionally, these factors, and others related to TGF-beta, appear to be involved in many processes of tissue development and repair. (J. Massague, Ann. Rev. Cell. Biol., 1990, 6:597-641).

Due to these diverse effects, TGF-beta-like proteins as a group may have potential for use as therapeutic agents for, inter alia, treating proliferative disorders. However, it is impossible to predict whether a TGF-beta-like protein having a known amino acid sequence will have an inhibitory effect on proliferating cells causing a particular disorder without also causing clinically unacceptable side effects, absent further empirical information. The proteins of the instant invention are TGF-beta-like based on their primary structures, and are capable of specifically inhibiting the proliferation of androgen-independent prostate cancer cells. However, absent additional empirical information, this specificity could not have been predicted based on amino acid sequence alone. Accordingly, the specific anti-prostate cancer activity of the instant TGF-beta-like proteins is most unexpected.

More specifically, this invention provides a recombinant protein which selectively inhibits the proliferation of androgen-independent prostate cancer cells when contacted therewith under suitable conditions, and whose amino acid sequence comprises the amino acid sequence shown in Figure 4 or functional derivative thereof ("first protein"). In one embodiment, the first protein is selected from the proteins designated "rGF-2H", "recombinant GF-2H", or "recombinant mature

GF-2H" discussed infra. In the preferred embodiment, the first protein is mature GF-2H.

This invention also provides an isolated protein which
5 selectively inhibits the proliferation of androgen-independent prostate cancer cells when contacted therewith under suitable conditions, and whose amino acid sequence consists of the amino acid sequence shown in Figure 4 or functional derivative thereof ("second protein"). In one embodiment, the second protein is
10 selected from the group consisting of "GF-2H" and "mature GF-2H" discussed infra. In the preferred embodiment, the second protein is mature GF-2H.

As used herein, a recombinant protein is a protein which is
15 obtained through the use of recombinant nucleic acid technology. Such recombinant protein's primary structure may be identical to that of its naturally occurring counterpart, or may contain additional amino acid residues. Additional residues may be present, for example, at the protein's N-terminal and/or C-
20 terminal end. An isolated protein means any protein having a chemical milieu containing less than 50% protein impurities. In one embodiment, the isolated protein has a chemical milieu containing less than 10% protein impurities, and in the preferred embodiment, less than 1% protein impurities.

25

As used herein, a protein which is a "functional derivative" of the protein whose sequence is shown in Figure 4 is a protein which possesses structural (i.e., amino acid sequence) and functional (i.e., selectively inhibits the
30 proliferation of androgen-independent prostate cancer cells) similarity thereto. Structurally similar proteins include, by way of example, proteins differing from the protein of Figure 4 by amino acid residue deletions, insertions, or conservative substitutions which do not substantially diminish the protein's
35 ability to selectively inhibit the proliferation of androgen-independent prostate cancer cells. An example of a conservative

amino acid substitution is the substitution of a leucine residue for an isoleucine residue.

As used herein, an "androgen-independent" prostate cancer cell is a prostate cancer cell which can survive (i.e. remain alive) as well as proliferate in the absence of androgens. As used herein, "proliferation" means the growth of cell population through cell division, and "selectively inhibit" androgen-independent cell proliferation means to reduce the in vitro rate 5 of androgen-independent cell proliferation by at least 50% without reducing the in vitro rate of HeLa cell proliferation by more than 20% at saturating conditions and as measured at a suitable point in time (e.g. 72 hours after initial contact with the instant protein). In the preferred embodiment, the in vitro 10 rate of androgen-independent cell proliferation is reduced by at least 90% without reducing the in vitro rate of HeLa cell proliferation by more than 10%. The rate of cell proliferation can readily be measured using known methods, such as the labeled 15 thymidine incorporation method described hereinbelow.

20

Suitable conditions under which the first and second proteins selectively inhibit the proliferation of androgen-independent prostate cancer cells when contacted therewith means physiological conditions, i.e. the chemical milieu surrounding 25 androgen-independent prostate cancer cells in situ which would permit the binding of an androgen-independent prostate cancer cell surface receptor to its corresponding naturally occurring ligand. Such conditions can readily be duplicated in vitro according to known methods.

30

This invention further provides a pharmaceutical composition for treating a subject afflicted with androgen-independent prostate cancer, comprising the first or second protein and a pharmaceutically acceptable carrier ("first 35 pharmaceutical composition").

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

As used herein, "subject" means any animal or artificially modified animal capable of developing or sustaining prostate cancer. Artificially modified animals include, but are not limited to, mice, rats, dogs, guinea pigs, ferrets, rabbits, and primates. In one embodiment, the artificially modified animal is a mouse suffering from induced prostate cancer. Examples of such animals are known in the art (N.M. Greenberg, et al., P.N.A.S. U.S.A., 92:3439-3443 (1995); T.C. Thompson, et al., Cancer 71: 1165-1171 (1993)). In the preferred embodiment, the subject is a human.

This invention further provides a method of treating a subject afflicted with androgen-independent prostate cancer which comprises administering to the subject a therapeutically effective dose of the first pharmaceutical composition.

As used herein, administering may be effected or performed using any of the various methods known to those skilled in the

art. The administering may comprise administering intravenously, intramuscularly, and subcutaneously.

A therapeutically effective dose of the first pharmaceutical composition is a dose sufficient to selectively inhibit the proliferation of androgen-independent prostate cancer cells in an afflicted subject. In the preferred embodiment, the treatment of an afflicted subject will comprise administering a plurality of therapeutically effective doses over a period of time. The dose and time period can be determined through known methods. In one embodiment, the therapeutically effective dose is from 0.1 ug/kg to 1 mg/kg, and in the preferred embodiment, the therapeutically effective dose is from 1 ug/kg to 100 ug/kg.

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This invention further provides a nucleic acid molecule consisting essentially of a nucleic acid sequence encoding the amino acid sequence of Figure 4 or functional derivative thereof.

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In one embodiment, the nucleic acid molecule is an RNA molecule. In another embodiment, the nucleic acid molecule is a DNA molecule (e.g. cDNA or genomic DNA). The DNA molecule may comprise the nucleotide sequence shown in Figure 1.

25

In an additional embodiment, the nucleic acid molecule is an expression vector. Numerous expression vectors may be employed. Such vectors, including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses, are well known in the art. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. In the preferred embodiment, the vector is pCI. Additionally, host cells, regulatory elements, and transfection methods suitable for use in connection with this invention are well known in the art.

In a further embodiment, the nucleic acid molecule is a vector suitable for use in gene therapy for treating a subject afflicted with androgen-independent prostate cancer. Gene 5 therapy vectors, and methods of delivering same, are well known in the art. Such delivery methods include, but are not limited to, delivery via adenovirus and cationic liposomes, as well as via naked DNA. Additionally, the instant vectors may be introduced into suitable cells ex vivo (e.g. peripheral blood 10 mononuclear cells (PBMC's)), which are then reintroduced into an afflicted subject. Methods for such ex vivo methods are known in the art.

This invention further provides a pharmaceutical 15 composition for treating a subject afflicted with androgen-independent prostate cancer, comprising the instant nucleic acid molecule suitable for use in gene therapy and a pharmaceutically acceptable carrier ("second pharmaceutical composition").

20 This invention further provides a method of treating a subject afflicted with androgen-independent prostate cancer which comprises administering to the subject a therapeutically effective dose of the second pharmaceutical composition.

25 A therapeutically effective dose of the second pharmaceutical composition is a dose effective to cause cells in the subject to produce and secrete the protein encoded by the nucleic acid molecule therein, which protein in turn selectively inhibits the proliferation of androgen-independent prostate 30 cancer cells in the subject. In one embodiment, the treatment of an afflicted subject will comprise administering a plurality of therapeutically effective doses over a period of time. The dose and time period can be determined through known methods. In one embodiment, the therapeutically effective dose of the 35 second pharmaceutical composition is from 10^4 to 10^{11} DNA molecules per subject, and in the preferred embodiment is from 10^5 to 10^{10} DNA molecules per subject.

This invention also provides a composition of matter which selectively kills androgen-independent prostate cancer cells when contacted therewith under suitable conditions, and which 5 comprises the first or second protein and a toxic moiety operably affixed thereto.

The toxic moiety may be a proteinaceous toxin. In one embodiment, the proteinaceous toxin is selected from the group 10 consisting of ricin, tetanus toxin, diphtheria toxin, and subunits and fragments thereof. The toxic moiety may also be a radionuclide. In another embodiment, the toxic moiety is an α - or β -emitting radionuclide including, but not limited to, ^{125}I , ^{131}I , ^{90}Y , ^{212}Bi . Methods for operably affixing toxic moieties to 15 proteins are well known in the art.

This invention also provides a pharmaceutical composition for treating a subject afflicted with androgen-independent prostate cancer, comprising the instant composition of matter 20 and a pharmaceutically acceptable carrier (the "third pharmaceutical composition").

This invention further provides a method of treating a subject afflicted with androgen-independent prostate cancer 25 which comprises administering to the subject a therapeutically effective dose of the third pharmaceutical composition.

As used herein, to "selectively kill" androgen-independent cells means to kill at least 50% of androgen-independent cells 30 in vitro without killing more than 20% of HeLa cells in vitro under saturating conditions as measured at a suitable point in time (e.g. 72 hours after initial contact with the instant composition). In the preferred embodiment, the instant composition kills at least 90% of androgen-independent cells in 35 vitro without killing more than 10% of HeLa cells in vitro. Cell

death can readily be measured using known methods including, for example, the Trypan Blue exclusion assay.

A therapeutically effective dose of the third pharmaceutical composition is a dose sufficient to selectively kill androgen-independent prostate cancer cells in an afflicted subject. In the preferred embodiment, the treatment of an afflicted subject will comprise administering a plurality of therapeutically effective doses over a period of time. The dose and time period can be determined through known methods. In one embodiment, the therapeutically effective dose is from 0.1 ug/kg to 1 mg/kg, and in the preferred embodiment, the therapeutically effective dose is from 1 ug/kg to 100 ug/kg.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental DetailsMaterials and Methods

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rIFN- γ was purchased from Boehringer and Mannheim ($> 2 \times 10^7$ units/mg). Expression vectors pCI, pcDNA3, and pSV2-dhfr were obtained from Promega, Invitrogen, and the American Type Culture Collection (ATCC), respectively. Cell culture media were obtained from GIBCO and BioWhittaker, and fetal bovine serum was obtained from Hyclone. Recombinant human TGF-beta was from R&D Systems. [3 H]thymidine was from DuPont NEN. S100 SartoBind cation membrane adsorber was from Sartorius. Chromatography columns and resins were from Pharmacia. Chemicals were from Fisher and Sigma.

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Cell lines and cell culture

All the cell lines were obtained from the ATCC. HeLa cells were maintained in MEM medium supplemented with 5% FBS, PC-3 and LNCaP cells in RPMI-1640 supplemented with 5% FBS, DU145 in EMEM supplemented with 10% FBS, HEL cells in RPMI supplemented with 10% FBS, HepG2 cells in MEM supplemented with 10% FBS and 1x non-essential amino acids, and BHK cells in DMEM supplemented with 5% FBS. Antibiotics (final concentration 50 ug/ml penicillin and 50 ug/ml streptomycin) were added to the culture media for all the cell lines.

Construction and screening of a subtractive cDNA library

HeLa cells (ATCC CCL185) were grown in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 u/ml penicillin and 50 μ g/ml streptomycin. HeLa cells were treated with recombinant human interferon- γ (IFN- γ) (Boehringer Mannheim) for 48 hours at a concentration of 2500 units/ml. Total RNA (RNAzol; Tel-Test) and poly (A)-selected RNA (FastTrack; Invitrogen) were isolated from mock and IFN- γ -treated HeLa cells following manufacturer's protocols.

For unidirectional cDNA library construction, cDNA's synthesized from 2 µgs of poly (A) RNA from untreated and IFN- γ -treated HeLa cells (48 hour treatment) were directionally cloned into the plasmids pGEM11zf (Promega) and pT7T3D18U (Pharmacia), respectively. Subtractive hybridization, or more specifically, sense-strand cRNA and antisense cDNA syntheses, hybridization, separation of single-stranded cDNA from double-stranded cDNA/RNA hybrids by hydroxyapatite column and PCR amplification followed by subtractive library construction were performed according to known methods (Usui et. al, J. Neurosci., 14(8):4915-4926, 1994). The PCR-amplified subtracted cDNA's were directionally cloned into pT7T3D (Pharmacia).

To screen the subtracted cDNA library, 1000 cDNA's from this library were randomly picked and sequenced (see below). Clone #15558 (aka 2H) was found 6 times (per 1000 cDNA's analyzed) and therefore considered as a cDNA to be putatively differentially regulated by IFN- γ .

20 Cloning, DNA sequencing, and Computer Analysis

All sequencing was done on the Applied Biosystems 373 or 377 DNA sequencer in a single pass using T7 primer according to the manufacturer's protocol. Vector sequences were removed before analysis using the Sequencer program (Gene Codes Corporation). Inserts greater than 20 bp were compared via Blast (Altschul et. al, J. Mol. Biol., 215:40, 1990) to GenBank (version 92) for gene identification and were compared with each other via FastA (Pearson & Lipman, PNAS, 85:2444, 1988) to calculate the frequency of cDNA appearance in the subtracted cDNA library. Sequences containing Alu elements were excluded from the analysis. Blast analysis of 2H (clone #15558) indicated a significant homology to several members of the TGF- β superfamily. Clone #15558 was not full-length (i.e., it did not contain the entire coding region) and thus anchor PCR (Frohman et. al, PNAS 85:8998-9002; 1988) was implemented using 2 µg of poly (A) RNA (extracted from HeLa cells treated for 48 hours with IFN- γ) as a source for cDNA template synthesis. Two

independent anchor PCR's were completed and the subsequent cDNA's were cloned and sequenced. An open reading frame was found with a stop codon upstream of this ATG indicating a full-length clone had been obtained.

5

Northern Blot Analysis

For RNA blots, 2 µg of poly (A) RNA extracted from HeLa cells (both mock control and HeLa cells treated with IFN- γ for 48 hours) were loaded per lane and subsequently resolved by electrophoresis on a 1.2% agarose, 1.2 M formaldehyde gel, transferred to a nitrocellulose membrane, and hybridized to a full-length 32 P-labeled 2H cDNA probe. In addition, this 32 P-labeled probe was hybridized to a human tissue RNA blot (Clontech).

15

Expression of rGF-2H

The entire coding region of GF-2H cDNA was subcloned into the EcoRI site of mammalian expression vector pCI (Promega) to generate the expression plasmid pCI-2H, which has a Kozak consensus sequence immediately upstream of the initiation codon ATG. DNA transfections were carried out by a modified calcium phosphate precipitation procedure (Graham and van der Eb, Virology 52:456 (1973)). For methotrexate (MTX) resistance, plasmid pSV2-dhfr (ATCC No. 37146; S. Subramani, et al., Mol. & Cell. Biol., Sept. 1981, pg. 854-864)) or pZem229 (European Patent Appl. No. 0 319 944 A2, filed July 12, 1988) was co-transfected with pCI-2H at a 1 to 4 ratio, and for G418 resistance, plasmid pcDNA3 (Invitrogen, #V790-20) was co-transfected with pCI-2H at a 1 to 4 ratio. Twenty-four hours after transfection, MTX (final concentration 1 uM) or G418 (final active concentration 1 mg/ml) was added to the culture media. Resistant colonies were randomly selected 10 days later. Serum-free condition culture media and cell lysates from selected clones were collected and subjected to Western blot analysis for expression and secretion of rGF-2H. Producing cell lines were established and maintained in 1 uM MTX or 1 mg/ml G418. Some of the MTX-resistant cell lines were subjected to 20

uM MTX to further amplify the transfected genes. The highest producing clone isolated was designated BHK-GF-2H #17 and used for the production of rGF-2H.

5 Purification of Recombinant Mature GF-2H

Serum-free condition medium from BHK-GF-2H #17 was collected and protease inhibitors were added at the following concentrations: 1 mM PMSF, 1 ug/ml leupeptin, 2 ug/ml pepstatin, 1 ug/ml aprotinin, and 1 mM EDTA. After centrifugation at 9000 rpm at 4°C for 20 minutes to remove cell debris, the supernatant was acidified with acetic acid (0.2 M final concentration) at 4°C overnight.

15 The supernatant was then loaded onto an S100 SartoBind cation membrane adsorber (Sartorius, #S100X) pre-equilibrated with 25 mM sodium phosphate pH 4.0 (Buffer A). The membrane adsorber was washed with Buffer A followed by Buffer A with 300 mM NaCl. rGF-2H was eluted from the membrane adsorber with 25 mM sodium phosphate pH 4.0 containing 500 mM NaCl and protease 20 inhibitors (1 mM PMSF, 1 ug/ml leupeptin, 2 ug/ml pepstatin, 1 ug/ml aprotinin, and 1 mM EDTA).

25 After the addition of saturated $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 1 M, the pool was applied to an 8 ml Butyl Sepharose column pre-equilibrated with 25 mM NaPO₄, pH 7.0 containing 1 M NaCl and 1 M $(\text{NH}_4)_2\text{SO}_4$. The column was then washed with the same buffer and eluted with a linear 1-0 M gradient of both NaCl and $(\text{NH}_4)_2\text{SO}_4$ in 25 mM NaPO₄, pH 7.0. Fractions containing rGF-2H were pooled and concentrated using 30 Centriprep-10 (Millipore).

The concentrated pool was applied to a Superdex-75 FPLC column (Pharmacia). The column was equilibrated and eluted with 20 mM Tris-HCl, pH 7.0, 100 mM KCl.

35

The fractions containing rGF-2H were applied to a Vydac C18 HPLC column (10- μ m particle size, 4.6 X 250 mm) equilibrated in 90% solvent A and 10% solvent B (solvent A contained 0.1% trifluoroacetic acid in water and solvent B was composed of 0.1 % trifluoroacetic acid in acetonitrile). The column was eluted with a 10% to 90% linear gradient of solvent B. Fractions containing pure rGF-2H were lyophilized to dryness and re-suspended in 25 mM NaPO₄, pH 7.0 and stored at -70°C in small aliquots.

10

Polyclonal Antibody to GF-2H

Peptide MHAQIKTSLHRLKPDTVPAPC, corresponding to residues 253 to 273 of pre-pro-GF-2H, was synthesized, coupled to keyhole limpet hemocyanin, emulsified in Freund's adjuvant, and injected 15 into rabbits. The rabbit antisera were titrated against the peptide by ELISA.

Gel Electrophoresis and Western Blot Analysis

SDS-polyacrylamide gel electrophoresis was performed 20 according to Laemmli, U.K. Nature, 227:680 (1970), and the gels were stained by either silver stain or Coomassie Brilliant Blue R-250. Western transfers were carried out as described (Towbin et al., PNAS, 76, 4350 (1979)). Immunostaining was performed using rabbit antibody against GF-2H peptide and horseradish 25 peroxidase-conjugated anti-rabbit antibody (Bio-Rad).

N-Terminal Amino Acid and Mass Spectrometry Analysis

N-terminal sequence analysis was performed using an Applied Biosystems 470A protein sequencer equipped with an on-line 30 phenylthiohydantoin analyzer (model 120A). Mass spectrometry analysis was performed using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) on a Vestec 2000 MALDI-TOF-MS with a Lumonics NAG laser tuned at 355nm.

35 Cell Proliferation Assay

Cells were seeded onto 96-well flat-bottom plates in 100 μ l of corresponding complete culture at a density of 5,000 to

10,000 cells/well. After incubation overnight at 37°C, 5% CO₂, culture media were replaced with 100 ul of fresh medium supplemented with either 1% or 5% FBS containing an indicated concentration of rGF-2H or TGF-beta. After further incubation at 5 37°C, 5% CO₂ for a designated time period, cells were labeled with [³H]thymidine (1 uCi/well) for 5 h at 37°C, 5% CO₂. At the end of incubation, the labeling media were removed, the cells were washed with PBS and incubated with 100 ul trypsin-EDTA at 10 37°C for 10 minutes. Cells were then transferred from the culture plate onto a 96-well glass fiber plate (Unifilter plate, Parkard) using a cell harvester. The filter plate was washed, dried at 55°C for 30 min., and each well was wetted with 40 ul of scintillation fluid (MicroScin-20, Parkard), and counted using a 96-well plate counter (TopCount, Parkard).

15

ResultsCloning of IFN- γ -Inducible Genes From HeLa Cells

The full-length clone of GF-2H-encoding DNA spans 1122 base pairs (Figure 1). An initiator methionine (base pairs 35-37) was identified by the following criteria: (a) there was an in-frame stop codon (base pairs 14-16) upstream of this methionine; (b) the amino acid sequence was consistent with Kozak's consensus sequence (Kozak, J. Biol. Chem., Vol. 266., No. 30, 19867-19870 (1991); and (c) this methionine was followed by a putative signal peptide.

A stop codon was found at base pairs 959-961, indicating the end of the open reading frame (Figure 1). This corresponds to an open reading frame of 927 base pairs. The 3' noncoding region contains a poly adenylation signal, AATAAA (base pairs 1100-1105), and two instability motifs, ATTTA (base pair 1075-1079), found in the 3'-noncoding regions of short-lived mRNAs.

20 Expression of GF-2H mRNA

Northern blot analysis was performed to determine the approximate length of the GF-2H transcript and to show the pattern of mRNA tissue expression. Northern blots were performed using a 1.2 kb fragment as a probe. Hybridization showed a major transcript at approximately 1.4 kb only in HeLa cells treated with IFN- γ and not in untreated HeLa cells (Figure 3A). Screening of different tissues showed that the GF-2H mRNA is detectable in placenta, prostate, liver, kidney, fetal lung, and fetal kidney (Figure 3B). The relative amounts of mRNA in placenta and prostate were greater than those in other tissues (Figure 3B).

Analysis of the Putative GF-2H Protein

The predicted GF-2H gene product is 308 amino acid residues long with a calculated molecular mass of 34.1 kDa. Both nucleotide and predicted protein sequences of GF-2H have been compared to those in sequence data banks. A Blast search of protein sequence databases revealed a significant level of homology between the putative GF-2H protein and the TGF-beta superfamily. The entire GF-2H protein shows 28.2% identity to the precursor form of human Gdf1, while the C-terminal one third of GF-2H protein shows 30.4% identity to the mature form of Drosophila dpp protein. When the sequence of the C-terminal one third of GF-2H protein was aligned with those of TGF-beta superfamily members for maximum similarity using the PILEUP program, it was observed that all of the nine conserved cysteins as well as some other conserved residues in the TGF-beta proteins (Hosaka, et al., J. Biol. Chem., Vol. 266, pp 12127-12130 (1991)) were present in the C-terminal one third of GF-2H protein.

Like most members of the TGF-beta superfamily, the GF-2H protein contains an N-terminal signal sequence and potential sites for N-linked and O-linked glycosylation. There is a cluster of basic residues which provide potential cleavage sites from which to generate the mature form from the precursor (Hosaka, et al. (1991)). Figure 2A shows the amino acid sequence of the immature GF-2H protein. Figure 2B shows a comparison between the amino acid sequence of mature GF-2H protein and those of other members of the TGF-beta family.

30 Expression of Recombinant GF-2H Proteins

To express recombinant GF-2H, the entire coding region of GF-2H cDNA was inserted into mammalian expression vector pCI to generate pCI-2H. Stable transfected BHK cell lines were obtained by co-transfected BHK tk-ts13 cells with plasmids pCI-2H and pSV2-dhfr or pZem229 containing an expression unit for mouse DHFR, which allowed for selection of transfectants with MTX. Expression of GF-2H by these cell lines was examined by Western

blot analysis using anti-GF-2H peptide antibody (data not shown). One of these cell lines, BHK-2H-#17, was used to further characterize expression of rGF-2H.

5 When the conditioned medium was fractionated on SDS-PAGE under reducing conditions and blotted with anti-GF-2H peptide antibody, a band with an apparent molecular weight of 13 kd was observed in BHK-2H-#17 cells (Figure 4, lane 5). This band was not seen in the control BHK cells (Figure 4, lane 4). On some
10 occasions, another band with an apparent molecular weight of 39 kd was also observed in the BHK-2H-#17 cells, but not in the control BHK cells (data not shown). When the conditioned medium was fractionated on SDS-PAGE under non-reducing conditions and blotted with anti-GF-2H peptide antibody, a band with an
15 apparent molecular weight of 25 kd was observed in BHK-2H-#17 cells (Figure 4, lane 3), but not in the control BHK cells (Figure 4, lane 2). These results suggest that GF-2H is expressed, processed to a smaller form, and secreted into the culture medium by BHK-2H-#17 cells. The secreted GF-2H is most
20 likely a disulfide-linked dimer (25 kDa) of the 13 kd protein. The 39 kd form is most likely to be the full-length GF-2H with some post translational modification, such as glycosylation, since the predicted molecular weight for the full-length GF-2H is 34 kd.

25

Purification and Characterization of Recombinant GF-2H

Conditioned medium from BHK-2H-#17 was used to purify the processed mature form of rGF-2H by a four-step purification protocol developed specifically for rGF-2H (see discussion
30 supra). Using this protocol, mature rGF-2H was purified to greater than 95% as judged by SDS-PAGE and silver stain analysis (data not shown).

When analyzed by SDS-PAGE under reducing conditions,
35 purified recombinant mature GF-2H was detected as a 13 kd band, while under non-reducing conditions it was detected as a 25 kd band (Figure 5). The molecular mass of mature rGF-2H was also

determined to be 25 kd by mass spectrum analysis (data not shown). N-terminal amino acid sequence analysis of purified recombinant mature GF-2H showed that the mature rGF-2H sequence begins at residue 197 of the full-length GF-2H (Figure 6). The calculated molecular weight of the polypeptide from residue 197 to 308 of the full-length rGF-2H is 12.3 kd. These results demonstrate that the mature rGF-2H contains the C-terminal portion of the full-length rGF-2H, residues 197 to 308, linked together by disulfide bond(s).

10

Effect of rGF-2H on Cell Proliferation

Purified rGF-2H was tested on a variety of human cell lines to examine its effects on cell proliferation. Cells were plated out, incubated with various concentrations of rGF-2H for 24 to 15 96 hours, and labeled with [³H]thymidine, as described supra. Human TGF-beta2 was also tested on these cell lines in parallel with rGF-2H in order to compare its effects with that of rGF-2H.

Recombinant GF-2H showed a dose- and time-dependent inhibition of [³H]thymidine incorporation by PC-3 cells, an androgen-independent prostate cancer cell line. This inhibitory effect was first observed after a 48-hour incubation period (Figure 6) and continued to be seen after a 96-hour incubation period, at which time significant cell death started to occur. 20 The maximum inhibition, approximately 90% compared with that of the control (without rGF-2H), was observed after a 72-hour incubation at 1.4×10^{-11} M rGF-2H (Figure 6). When TGF-beta was tested on PC-3 cells under the same conditions, a similar dose- and time-dependent inhibition to that of rGF-2H was also 25 observed. However, the maximum inhibition seen with TGF-beta was only 52% when compared to the control (Figure 7). Similar to PC-3 cells, dose- and time-dependent inhibition of [³H]thymidine incorporation by rGF-2H and TGF-beta were seen with DU145 cells, another androgen-independent prostate cancer cell line (Table 30 1). The maximum inhibition by rGF-2H and TGF-beta was 60% and 35 80%, respectively, compared with that of controls (data not shown).

Table 1
Effects of rGF-2H on Proliferation of Selected Human Cell Lines

	<u>Cell Line</u>	<u>rGF-2H</u>	<u>TGF-beta</u>
5	PC-3 (androgen-independent prostate carcinoma)	inhibition	inhibition
10	DU145 (androgen-independent prostate carcinoma)	inhibition	inhibition
15	LNCaP (androgen-dependent prostate carcinoma)	no effect	inhibition
	HepG2 (hepatoma)	no effect	inhibition
	HEL (erythroleukemia)	no effect	inhibition
20	HeLa (cervix epitheloid carcinoma)	no effect	slight inhibition
	Caov-3 (ovary adeno-carcinoma)	no effect	no effect

As summarized in Table 1, rGF-2H had no effect on [³H]thymidine incorporation by LNCaP, Hep G2, or HEL cells. In contrast, TGF-beta inhibited [³H]thymidine incorporation by these cells. Neither rGF-2H nor TGF-beta showed significant effects on [³H]thymidine incorporation by HeLa and Caov-3 cells. In short, rGF-2H was shown to specifically inhibit the proliferation of androgen-independent prostate cancer cells, unlike TGF-beta which shows inhibitory behavior on additional cell lines.

What is claimed is:

1. A recombinant protein which selectively inhibits the proliferation of androgen-independent prostate cancer cells when contacted therewith under suitable conditions, and whose amino acid sequence comprises the amino acid sequence shown in Figure 4 or a functional derivative thereof.
- 10 2. An isolated protein which selectively inhibits the proliferation of androgen-independent prostate cancer cells when contacted therewith under suitable conditions, and whose amino acid sequence consists of the amino acid sequence shown in Figure 4 or a functional derivative thereof.
- 15 3. A pharmaceutical composition for treating a subject afflicted with androgen-independent prostate cancer, comprising the protein of claim 1 or 2 and a pharmaceutically acceptable carrier.
- 20 4. A method of treating a subject afflicted with androgen-independent prostate cancer which comprises administering to the subject a therapeutically effective dose of the pharmaceutical composition of claim 3.
- 25 5. The method of claim 4, wherein the subject is a human.
6. A nucleic acid molecule consisting essentially of a nucleic acid sequence encoding the amino acid sequence of Figure 4 or functional derivative thereof.
- 30 7. The nucleic acid molecule of claim 6, wherein the nucleic acid molecule is an RNA molecule.
- 35 8. The nucleic acid molecule of claim 6, wherein the nucleic acid molecule is a DNA molecule.

9. The nucleic acid molecule of claim 8, wherein the DNA molecule comprises the nucleotide sequence shown in Figure 1.
- 5 10. The nucleic acid molecule of claim 7, wherein the nucleic acid molecule is an expression vector.
11. The expression vector of claim 10, wherein the expression vector is the vector designated pCI.
- 10 12. The nucleic acid molecule of claim 7 or 8, wherein the molecule is a vector suitable for use in gene therapy for treating a subject afflicted with androgen-independent prostate cancer.
- 15 13. A pharmaceutical composition for treating a subject afflicted with androgen-independent prostate cancer, comprising the nucleic acid molecule of claim 12 and a pharmaceutically acceptable carrier.
- 20 14. A method of treating a subject afflicted with androgen-independent prostate cancer which comprises administering to the subject a therapeutically effective dose of the pharmaceutical composition of claim 13.
- 25 15. The method of claim 14, wherein the subject is a human.
16. A composition of matter which selectively kills androgen-independent prostate cancer cells when contacted therewith under suitable conditions, and which comprises the protein of claim 1 and a toxic moiety operably affixed thereto.
- 30 35 17. The composition of matter of claim 16, wherein the toxic moiety is a proteinaceous toxin selected from the group consisting of ricin, tetanus toxin, diphtheria toxin, and subunits and fragments thereof.

18. The composition of matter of claim 16, wherein the toxic moiety is a radionuclide selected from the group consisting of ^{125}I , ^{131}I , ^{90}Y , and ^{212}Bi .
- 5 19. A pharmaceutical composition for treating a subject afflicted with androgen-independent prostate cancer, comprising the composition of matter of claim 16 and a pharmaceutically acceptable carrier.
- 10 20. A method of treating a subject afflicted with androgen-independent prostate cancer which comprises administering to the subject a therapeutically effective dose of the pharmaceutical composition of claim 19.
- 15 21. The method of claim 20, wherein the subject is a human.

FIG. 1NUCLEOTIDE SEQUENCE OF GF-2H

1	CGAGTCCCAG	CTCTGAGCCG	CAACCTGCAC	AGCGATGCC	GGCCAAGAAC
51	TCAGGACGGCT	GAATGGCTCT	CAGATGCCTCC	TGGTGTGCTGCT	GGTGCTCTCG
101	TGGCTGCCGG	ATGGGGCGC	CCTGTCTCTG	GCCGAGGCCAAG	GCCGGGCCAAG
151	TTTCCCCGG	CCCTCAGAGT	TGCACTCCGA	AGACTCCAGA	TTCGGAGAGT
201	TGCGGAAACG	CTACGAGGAC	CTGCTAACCA	GGCTGCGGGC	CAACCAGAGC
251	TGGGAAGATT	CGAACACCGA	CCTCGTCCC	GCCCCCTGCAG	TCCGGATACT
301	CACGGCCAGAA	GTGGGGCTTG	GATCCGGGG	CCACCTGCAC	CTGGGTATCT
351	CTCGGGGGC	CCTTCTTGCG	GGGCTCCCC	AGGCTCCCC	CCTTCACCGG
401	GCTCTTCC	GGCTGTCCCC	GACGGCGTCA	AGGTCTGTTGG	ACGTGACACG
451	ACCGCTGGG	CGTCAGCTA	GCCCTTGCAAG	ACCCCAGGGC	CCC GGCTGC
501	ACCTGGGACT	GTCGGGGCC	CCGTCGGCAGT	CGGACCAAAC	GCTGGCAGAA
551	TCTCGTCCG	CACGGCCCCA	GCTGGAGTTG	CACTTGGGGC	CGCAAGCCGC
601	CAGGGGGCGC	CGCAGAGGCC	GTGGGGGCAA	CGGGGACCCAC	TGTCCGGCTCG
651	GGCCCCGGCG	TTGGCTGGCG	CTGGCACACGG	TCCGGGGGTC	GCTGGAAAGAC
701	CTGGGCTGGG	CCGATTGGGT	GCTGTGCCCA	CGGGGAGGTGTC	AAGTGACCAT
751	GTGCATCGGC	GCGTGCCGA	GCCAGTTCCG	GGCGGGCAAAC	ATGCACGGCGC
801	AGATCAAGAC	GAGCCTGCAAC	CGCCTGAAGC	CGGACACGGT	GCCAGGGCCC
851	TGCTGGTGC	CGGCCAGCTA	CAATCCCAGT	GTGCTCATTC	AAAAGACCGA
901	CACGGGGGT	TGGCTCCAGA	CCTATGATGA	CTTGTAGCC	AAAGACTGCGC
951	ACTGCATATG	AGCAGTCCCTG	GTCCTTCAC	TGTGCACCTG	CGCGGGGAG
1001	GCGACCTCAG	TTGGTCCCTGC	AGGCTGAATG	GGCTCAAGGT	TCCTGAGACA
1051	CCCGGATTCCCT	GCCCCAAACAG	CTGTATTAT	TTAAACTCT	GATGATAAAA
1101	ATAAAGCTTG	TCTTGAACCTG	TT		

FIG. 2A

H-TGF-B2-P	1	ALDAAYCFCR -	-NVQDNCCLRAPLYIDFKRDLGWK-	31
H-TGF-B3-P	1	ALDTNYCFCR -	-NLEENCCVRPLYIDFRQDLGWK-	31
B-TGF-B1-F	1	ALDTNYCFS -	-STEKNCCVRQLYIDFRKDLGWK-	31
C-TGF-B4-F	1	DLDTNYCFFG -	-DEKNCCVRPLYIDFRKDLGWK-	33
F-TGF-B5-P	1	GVGOEYCFG -	-NNGPNCVYKPLYINFRKDLGWK-	31
ACTIVE2H2	1	ARNGDHCPGLG -	-PGRCRLHTVRAASLEDLGWAD	31
H-TGF-B2-P	32	WIHEPKGYNANFCAGACPYLWSSDTQHSRVLSLY	65	
H-TGF-B3-P	32	WVHEPKGYYANFCSGPCPYLRSADTTIHSSTV	65	
B-TGF-B1-F	32	WIHEPKGYHANFCCLGPCPYIWSSLDTQYSKV	65	
C-TGF-B4-F	34	WIHEPKGYMANFCMGPCPYIWSAADTQYTKV	67	
F-TGF-B5-P	32	WIHEPKGYEANYCLGNCPYIWSMDTQYSKV	65	
ACTIVE2H2	32	WVLSPREVQVTPMCIGACPSQFRAANMHAQIKTS	65	
H-TGF-B2-P	66	NTINPEASASPCCVSQDLEPLTILYYIGKTPKIE	99	
H-TGF-B3-P	66	NTLNPEASASPCCVPQDLEPLTILYYVGRTPKVE	99	
B-TGF-B1-F	66	NQHNPGASAAPCCVPQALEPLPIVYYVGRRKPKVE	99	
C-TGF-B4-F	68	NQHNPGASAAPCCVPQTLDPPLPIYYVGRNVRVE	101	
F-TGF-B5-P	66	NQNNPGASIAPCCVPDVLEPLPIYYVGRRTAKVE	99	
ACTIVE2H2	66	HRLRKDPTVPAPCCVPASYNPMVLIQKIDTGVSLAQ	99	
H-TGF-B2-P	100	QLSNMIVKSCKCS	112	
H-TGF-B3-P	100	QLSNMIVKSCKCS	112	
B-TGF-B1-F	100	QLSNMIVRSCKCS	112	
C-TGF-B4-F	102	QLSNMIVRACKCS	114	
F-TGF-B5-P	100	QLSNMIVRSCKCS	112	
ACTIVE2H2	100	TYDDLLAKDCHC	112	

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FIG. 2BAMINO ACID SEQUENCE OF GF-2H

1 MPGQELRTLN GSQMLLVLLV LSWLPHGGAL SLAEASRASF PGPSELHS
51 SRFRELKRY EDLLTRLRA N QSWEDSNTDL VPAPAVRLLT PEVRLGSGGH
101 LHLRISRAAL PEGLPEASRL HRALFRLSPT ASRSWDVTRP LRQLSLARP
151 QAPALHLRLS PPPSQSDQLL AEASSARPQL ELHLRPQAAR GRRRARANG
201 DHCPLGPGR CRLHTVRASL EDLGWADWVL SPREVQVTM C IGACPSQFRA
251 ANMHAQIPTS LHRLKPDTVP APCCVPASYN PMVLIQKTDT GVSLQTYDDL
301 LAKDCHCI

FIG. 3A

PURIFICATION OF GF-2H

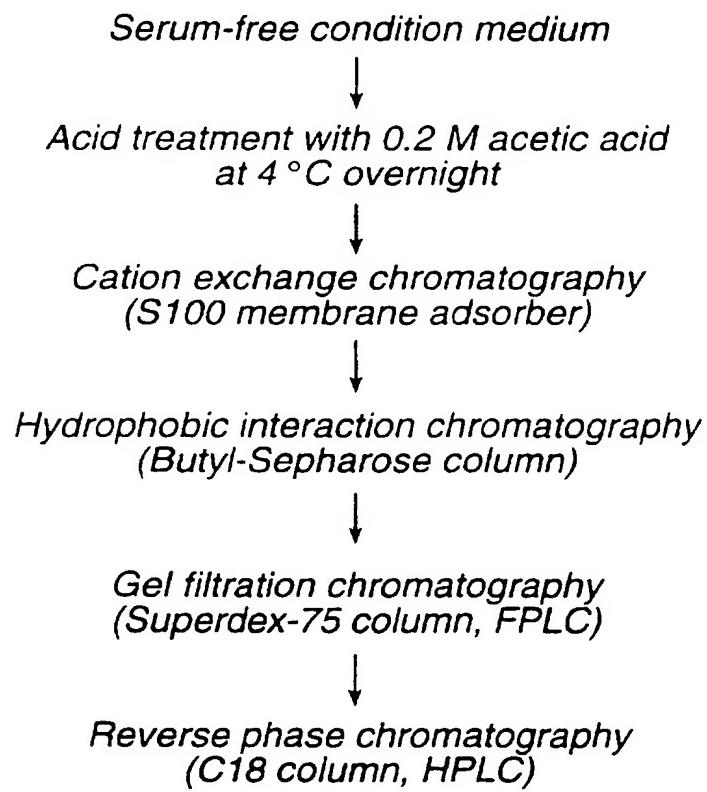
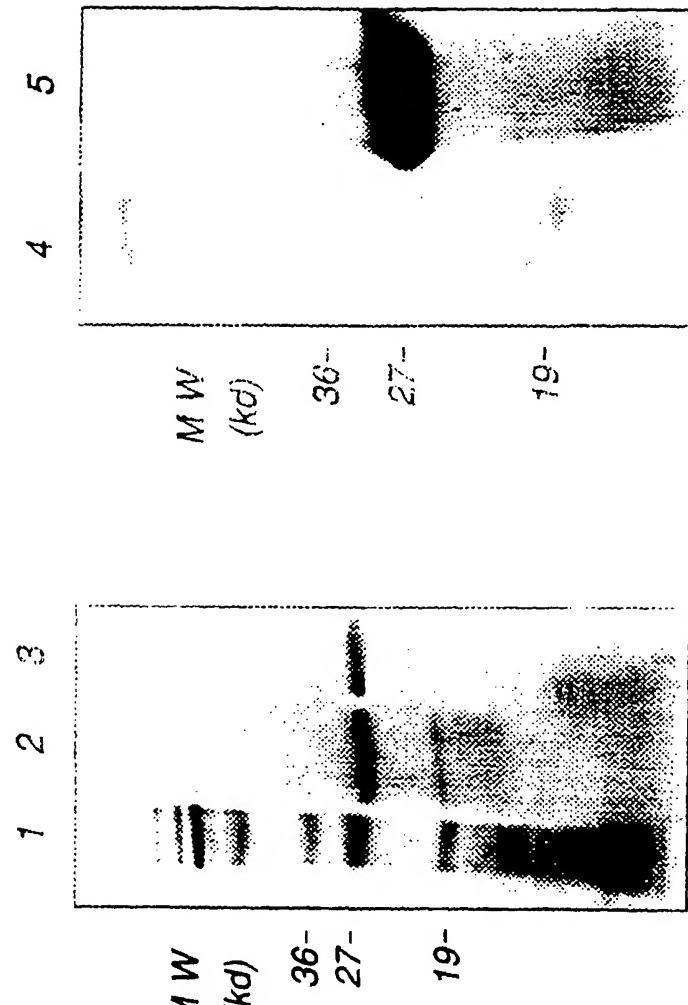


FIG. 3B

Purification of recombinant GF-2H



- 1&4. molecular weight standards
 2. silver strain of GF-2H after FPLC purification
 3. silver strain of GF-2H after HPLC purification
 5. Western blot of GF-2H after HPLC purification
 with anti 2H peptide antibody

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FIG. 4AMINO ACID SEQUENCE OF MATURE GF-2H

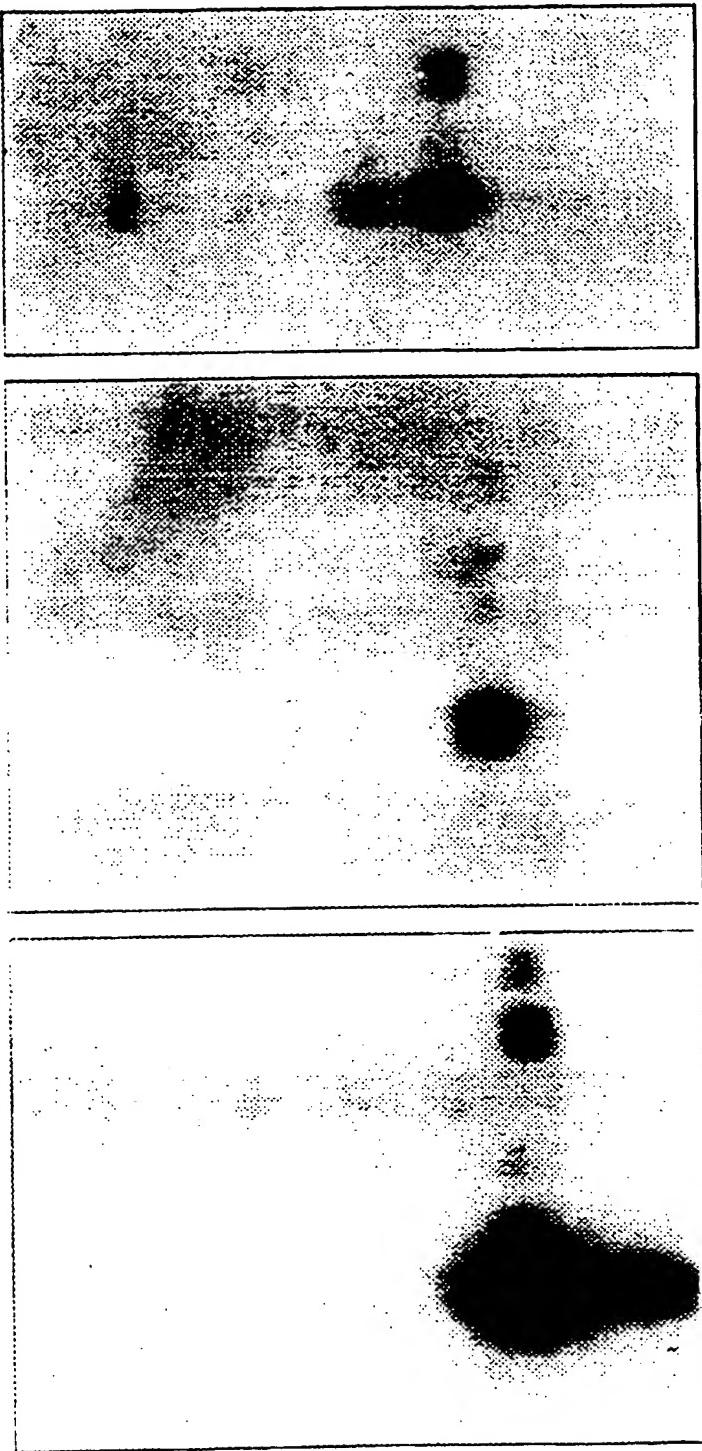
1 ARNGDHCPLG PGRCCRLHTV RASLEDLGWA DWVLSPREVQ VTMCIGACPS
51 QFRAANMHAQ IKTSLHRLKP DTVPAPCCVP ASYNPMVLIQ KTDTGVSLQT
101 YDDLLAKDCH CI

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FIG. 5

Northern blot of GF-2H

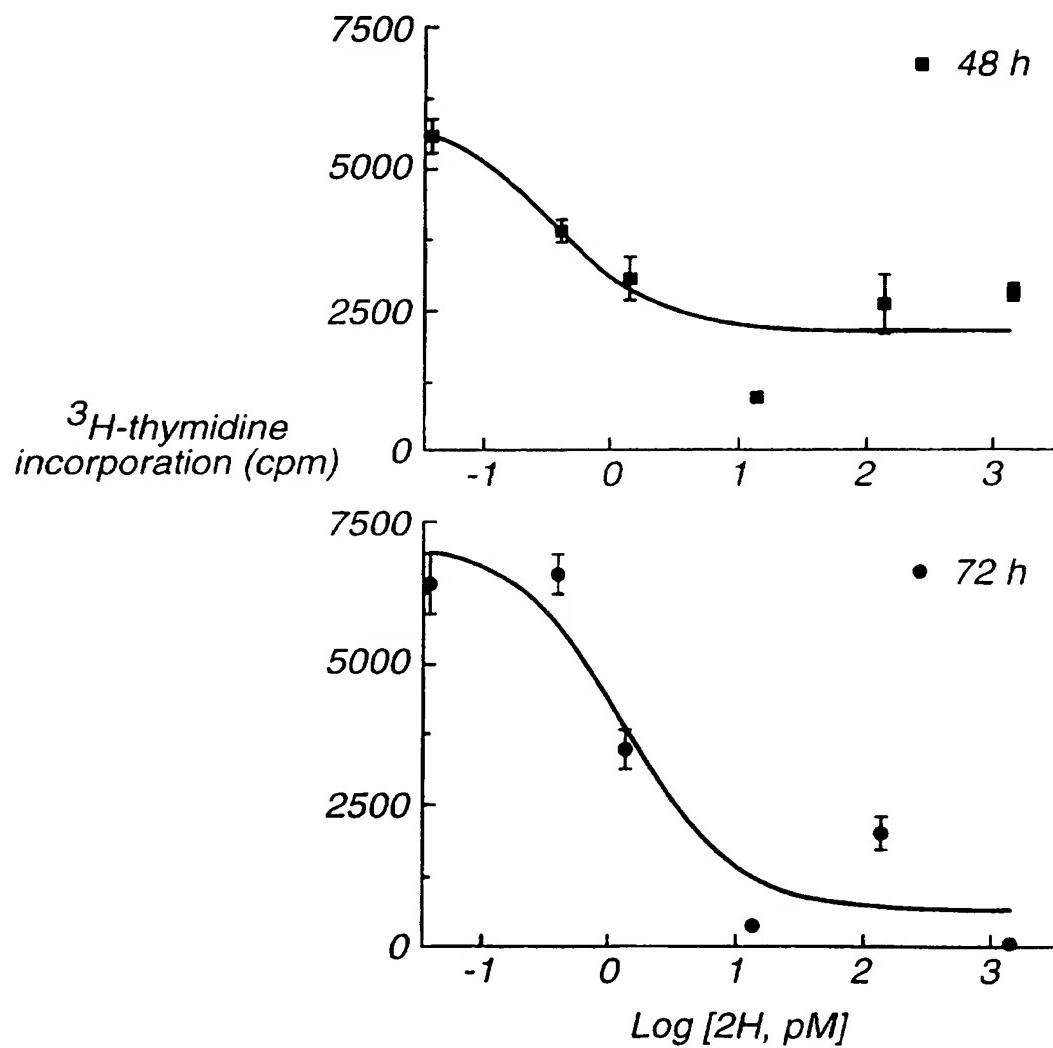
placenta	skeletal muscle	pancreas	prostate	small intestine	Kidney
brain	liver	thymus	thymus	leukocyte	lung
heart	lung	testes	colon	brain	liver



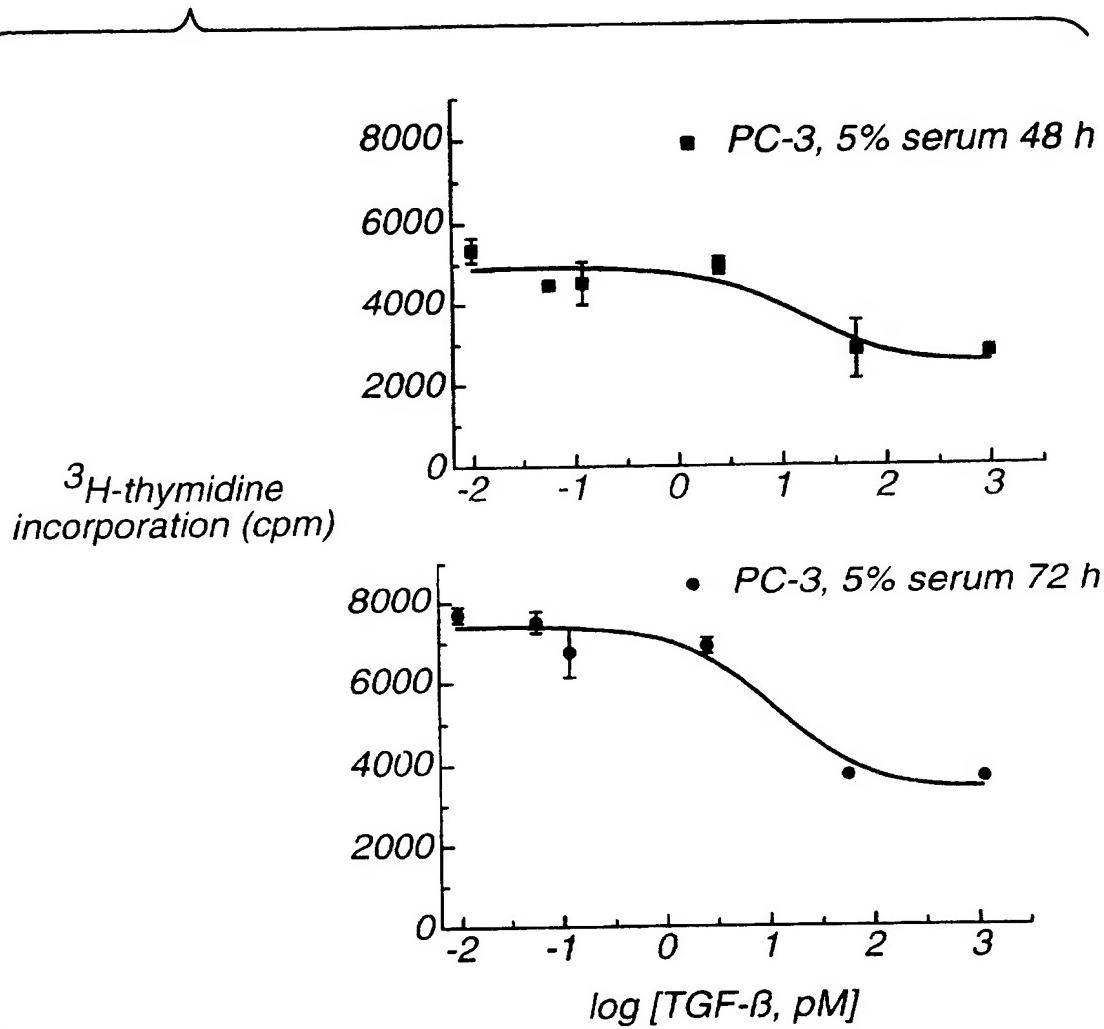
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Fetal Tissues

Adult Tissues

FIG. 6INHIBITION OF PC-3 CELL PROLIFERATION BY GF-2H

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FIG. 7

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/16191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C07K14/495 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 9549 Derwent Publications Ltd., London, GB; Class B04, AN 95-380074 XP002054319 & JP 07 258 293 A (ASAHI KASEI KOGYO KK) , 9 October 1995 see abstract ---	1-21
X	WO 96 18730 A (HUMAN GENOME SCIENCES, INC., USA) 20 June 1996 see page 16, paragraph 2; figure 1 ---	1-21
P,X	WO 97 00958 A (ST VINCENT'S HOSPITAL SYDNEY LTD) 9 January 1997 see figure 1 see page 3, line 26 - line 27 see page 5, line 5 - line 6 ---	1-21
	-/-	

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Patent family members are listed in annex.

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1

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 97/16191

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	DATABASE WPI Section Ch, Week 9548 Derwent Publications Ltd., London, GB; Class B04, AN 95-370476 XP002054320 & JP 07 250 688 A (SAGAMI CHEM RES CENTRE) , 3 October 1995 see abstract -----	1-21

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WO 9736926 A	09-10-97	NONE	
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